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Bacteria from nodules of wild legume species: Phylogenetic diversity, plant growth promotion abilities and osmotolerance

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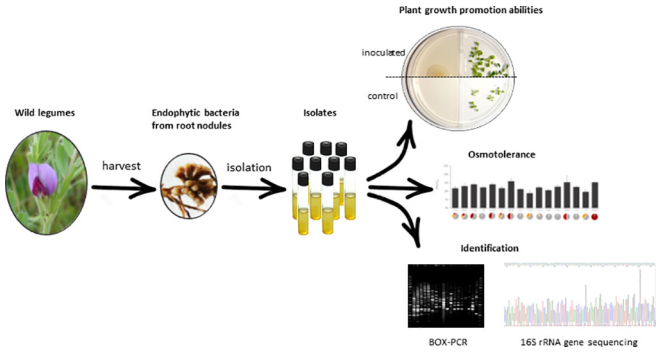
HIGHLIGHTS

- The majority of bacteria were *Flavobacterium* and *Pseudomonas* and not rhizobia.
- Bioclimatic conditions and host species do not influence bacteria osmotolerance.
- Wild legumes can be rich sources of bacteria with plant growth promotion abilities.
- Strains can be inoculants of legumes and non-legumes.

GRAPHICAL ABSTRACT



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ABSTRACT

The demand for food with high nutritional value that can sustain the growth of human population while safeguarding sustainability deserves urgent attention. A possible strategy is the inoculation of crops with plant growth promoting (PGP) bacteria. Plants are naturally colonized by bacteria that can exert beneficial effects on growth and stress tolerance. N_2 fixation by rhizobia in the root nodules of legumes is a well-known PGP effect. These bacteria can be used as inoculants to boost legumes productivity and can be especially interesting if they are able to survive to abiotic stresses, such as drought. Herein we report the phylogenetic diversity of bacteria colonizing the root nodules of several wild legume species, from four geographic locations in Portugal with different bioclimates. Interestingly, the vast majority of strains belonged to *Flavobacterium*, *Pseudomonas* and other genera apart from rhizobia. PGP abilities other than N_2 fixation (production of indol acetic acid, siderophores and volatile organic compounds) and osmotolerance were screened. Location and host plant species did not influence PGP abilities and osmotolerance. Taken together, results evidenced that bacterial strains from wild legumes displaying PGP abilities and osmotolerance can be regarded as good candidates for inoculants of a broad range of hosts, including non-legumes.

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1. Introduction

Legumes are an important protein source for human consumption (Ben Romdhane et al., 2009; Singh and Singh, 1992) and animal feed (Hanbury et al., 2000). This is particularly true for developing countries

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(Iqbal et al., 2006) and semi-arid and tropical regions (Singh and Singh, 1992). Plant productivity is affected by drought, which is a key constraint (Vurukonda et al., 2016), especially for grain legumes (Tuteja et al., 2012). For southern Europe, the area where this study was undertaken, the number of extreme drought events is predicted to double by the 2090s, while the duration of these events is expected to increase by a factor of six (IPCC, 2007). The increase of temperatures will also be observed (IPCC, 2007), along with a decrease in summer rainfall, which will both contribute to higher evaporation and thus lower summer soil moisture and more frequent and intense droughts (IPCC, 2007). However, an ever-growing global population and limited arable land demand an increase in crop yields, while safeguarding sustainability. Thus, environmentally friendly solutions that boost agricultural productivity with a low cost are required. One possible strategy to boost plant productivity, with lower impact on the environment than the use of inorganic fertilizers and pesticides, is the inoculation of plants with plant growth promoting rhizobacteria (PGPR). These bacteria colonize plant roots and can have several beneficial effects for the plant, namely the synthesis of compounds that stimulate plant growth, increase nutrients bioavailability, relieve abiotic stress or work as biocontrol agents of plant pathogenic organisms (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009).

Among the capabilities of plant growth promoting bacteria are the production of phytohormones such as indol acetic acid, which stimulates lateral root development (Ahemad and Kibret, 2014), the production of siderophores, which increase iron availability for plants (Ahemad and Kibret, 2014), and the emission of volatile organic compounds (VOCs) which can promote growth (Ryu et al., 2003; Bailly and Weisskopf, 2012). Several beneficial effects of PGPR can be associated with enhancement of plant's tolerance to drought, such as the production of phytohormones, alteration in root morphology, induction of osmolytes accumulation and emission of volatile organic compounds (Vurukonda et al., 2016; Schulz and Dickschat, 2007; Garnica-Vergara et al., 2015; Cho et al., 2008; Gutiérrez-Luna et al., 2010; Fincheira et al., 2016). The effects of bacterial VOCs on plant growth (Ryu et al., 2003) can have important ecological and economic roles since VOCs can travel the soil easier than non-volatile molecules (Schmidt et al., 2015).

Extensive research has been undertaken concerning nitrogen fixing rhizobia, which are harbored in nodules of crop legume species. However, in wild areas plant growth promotion effects of nitrogen fixing and endophytic (rhizobia and non-rhizobia) bacteria in general have been overlooked, despite their influence in wild legumes growth. In fact, wild legumes are commonplace in arid areas, and might be more tolerant to stress than crop legumes (Zahran, 2001). Thus, knowledge on the bacteria living inside wild legume nodules is important since these can be a source of interesting PGPR which can be used both in agricultural and environmental contexts as inoculants for legume and non-legume crops. Rhizobia are among the most well studied endophytic bacteria in legumes, however other bacteria can be found in legume root nodules (Martínez-Hidalgo and Hirsch, 2017). The diversity of plants that exist in an ecosystem is, among other factors, influenced by the prevailing climatic conditions. This diversity can in theory have also an effect on the microbiome associated to plants. Therefore, the more diverse the sites are the more likely it is to obtain bacteria with different plant promotion traits, which can be used as inoculants.

Improvement of legumes productivity in soils affected by drought can be aimed by selecting tolerant legume varieties but also by rhizobial inocula that are also adapted to these conditions (Coba de la Peña and Pueyo, 2012). Although little is known about the microbiome of wild legumes, it is expected that these plants harbor different bacteria in their root nodules when compared with crop legumes. This bacterial diversity can be a source of new inoculants to be used in agriculture. Along with plant growth promotion (PGP) abilities, osmotolerance of endophytic bacteria might depend on the site where the bacteria are collected from and is also a feature to be looked for when selecting inoculants.

Taking into consideration that climate change also influences legume species diversity and their symbionts due to altered rainfall profiles, warming, changes in soil properties and wildfires (Reverchon et al., 2012), the aim of this study was to explore the diversity of bacteria harbored in the root nodules of legume species growing in wild areas of Portugal, an approach that can provide new bacteria strains with particular PGP abilities, when compared to bacteria isolated from cultivated legume species. With this objective in mind, four sampling sites with different geographic and climatic conditions in Portugal were selected. We hypothesized that different sites would present different legume species, and consequently different bacteria being harbored in the root nodules. From each site the dominant legume species were collected and the bacteria colonizing the root nodules isolated. Isolates were identified by 16S rRNA gene sequencing and their tolerance to drought and plant growth promotion abilities (production of indol acetic acid, production of siderophores and emission of volatiles that induce plant growth) were screened.

2. Materials and methods

2.1. Plant sampling and identification

Specimens from different legume species were sampled from four sites in Continental Portugal in spring 2015. The geographic location of each site is presented in Fig. 1. Sites were chosen based on different bioclimatic conditions. Murtosa (MT) is located in inferior humid and inferior mesomediterranic (ambrotype/thermotype), Vale de Cambra (VC) in inferior hyperhumid and inferior mesotemperate, Alvito (AV) in superior dry and inferior mesomediterranean, and finally Aljustrel (AT) in inferior dry and superior termomediterranean (Mesquita, 2005). Fifteen wild legume plants at the flowering stage were collected in each site. At least one representative of each legume species was selected. The remaining plants were collected randomly. The full list of collected plant species on each site yielding bacterial isolates that were identified is provided in Supplementary Table 1. The aerial parts of plants were preserved in the herbarium of the University of Aveiro and identified according to *Flora Iberica* (Castroviejo, 2012).

2.2. Isolation of bacteria

Five nodules were randomly picked from each plant. Endophytic bacteria from root nodules were isolated following the method described by Somasegaran and Hoben (1994), with some modifications. Nodules were surface sterilized by soaking in 96% ethanol for 5 s, and then immersed for 2 min in a 30% hydrogen peroxide solution. Nodules were then rinsed twice in sterile deionised water and crushed. The macerate was streaked onto yeast extract mannitol (YMA) plates, containing 1 g mannitol, and supplemented with Congo red (CR). Morphologically distinct single colonies evidencing low absorption of CR were further re-streaked onto YMA + CR plates and allowed to grow at 26 °C. Following this methodology, 180 isolates were obtained. A loopful of each isolate was used to inoculate a tube of 5 mL of YMB. Tubes were incubated at 26 °C and 500 μL of the resulting cultures were transferred to microtubes. These cultures were preserved by adding 500 μL of a sterile solution of 30% glycerol, vortexing and storing at −80 °C.

2.3. Bacteria identification

2.3.1. PCR-based fingerprinting

Since bacteria were isolated from multiples nodules of the same plant, isolates were typed using BOX-PCR to screen for isolates with unique fingerprints, before proceeding to 16S rRNA gene amplification. This way the identical genotypes can be identified and only a representative isolate of each fingerprint pattern proceeds to identification by 16S RNA gene sequencing. Isolates were inoculated onto YMA + CR

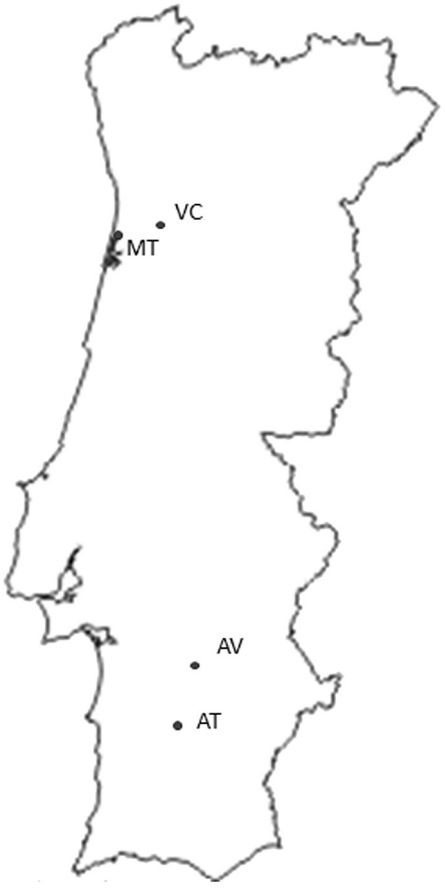

Fig. 1. Map of Continental Portugal with the four harvesting sites marked: Aljustrel (AT); Alvito (AV); Murtosa (MT); and Vale de Cambra (VC).

and single colonies were used to prepare a bacterial suspension in 100 μL of autoclaved deionised water. Each PCR reaction tube contained a mixture of 1 μL bacterial suspension, 1 μL BOXA1R primer (5′-CTACGG CAAGGCGACGCTGAC-3′; Versalovic et al., 1994), which was diluted in sterile Milli-Q water to 10 μmol/μL, 6.25 μL NZYTech 2× Taq Green Master Mix (NZYTech), and autoclaved Milli-Q water to fill up the mixture to 25 μL. Amplification was performed applying one cycle at 95 °C (7 min), 30 cycles at 94 °C (1 min), 53 °C (1 min), 65 °C (8 min), and a final cycle at 65 °C (16 min). GelCompar II (Applied Maths, Belgium) was used to calculate the Pearson correlation coefficient and analyze the clusters formed applying the unweighted pair group method with arithmetic mean (UPGMA). A representative isolate of each distinct fingerprint was selected randomly. This procedure yielded 100 representative isolates.

2.3.2. 16S rRNA gene amplification and phylogenetic analysis

The 16S rRNA gene from the representative isolates obtained in Section 2.2 was amplified using primers 27f (5′-AGAGTTTGATCCTGGC TCAG-3′; Lane, 1991) and 1492r (5′-GGTTACCTTGTTACGACTT-3′; Lane, 1991) and the NZYTaq 2× Green Master Mix (NZYTech, Portugal) in 25-μL tubes by applying one cycle at 94 °C (5 min), 30 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (1.5 min), and a final cycle at 72 °C (10 min). PCR products were sent to GATC Biotech (Germany) for sequencing. Each sequencing reaction tube contained 5 μL PCR product, 2.5 μL 27f primer and 2.5 μL autoclaved Milli-Q water. Sequences were edited with the software FinchTV V1.4.0 (Geospiza, USA). A BLAST search against the GenBank database was performed to identify the bacteria to genus level. The partial 16S rRNA gene sequences from the representative isolates were deposited in GenBank (MH236648-MH236745;MH594731;MH594732).

2.4. Plant growth promotion abilities

2.4.1. Production of siderophores

To assess the ability of bacteria to produce siderophores, strains (100) that were identified by 16S rRNA gene sequencing were grown for six to ten days on YMA medium supplemented with a chrome azurol S (CAS) solution, which consisted of 1.21 mg mL^{-1} CAS, 0.1 mM $FeCl_3 \cdot 6H_2O$ and 1.82 mg mL^{-1} hexadecyltrimethylammonium bromide (HDTMA). The presence of an orange halo around the colonies was considered as positive for siderophore production (Alexander and Zuberer, 1991). Data are presented as a ratio between the diameter of the halo and the diameter of the colonies.

2.4.2. Production of indol acetic acid (IAA)

To quantify the production of indol acetic acid (IAA), strains were grown in 5 mL tubes of yeast-mannitol broth (YMB) supplemented with 100 μg/mL of tryptophan, the precursor of IAA. Cells were grown at 26 °C, 150 rpm, until 1.0 optical density at 600 nm or maximum growth after five days was reached. After centrifugation at 10000*g* 4 °C, the supernatant was collected and 500 μL of supernatant reacted with 200 μL Salkowski reagent (0.5 M $FeCl_3 \cdot 6H_2O$, 35% $HClO_4$) for 10 min at room temperature. The color was then measured at 530 nm using a spectrophotometer. The concentration of IAA was determined using IAA (Sigma) as standard (Asghar et al., 2002). Data are presented in concentration of IAA (μg/mL) normalized by the optical density (OD) of each strain.

2.4.3. Growth promotion of a non-legume through the emission of volatiles

To screen for growth promotion of a non-legume the setup used by Ryu et al. (2003) was followed, with modifications. *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds were surface sterilized by immersion in 70% ethanol, followed by 100% ethanol, and allowed to dry. Seeds were then transferred to plates containing half-strength Murashige and Skoog medium, pH 5.7, supplemented with 1.5% sucrose and 0.8% agar, which were vernalized at 4 °C. After 2 days, plates were placed in a growth chamber set for 16 h light/8 h dark, 25 °C and 85% humidity, and allowed to germinate for 3 days. For the trials, center divided plates containing YMA in side I and Murashige and Skoog (MS) in side II were used. A strain per plate was spotted in side I. In side II of the plate, ten seedlings were planted. Non-inoculated plates were used as controls. Plates were sealed with parafilm and placed randomly in the chamber during 14 days. At the end of the trials, *A. thaliana* total leaf area and total fresh weight were recorded. Total leaf area was measured using Easy Leaf Area (Easlon and Bloom, 2014). Data are presented as means of the ratios between the total fresh weight or leaf area of strains isolated from each host species and the controls.

2.5. Bacteria osmotolerance

The osmotolerance of rhizobacteria strains was screened in YEM (Somasegaran and Hoben, 1994) with different polyethylenoglycol 6000 (PEG) concentrations: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0 and 30%. For each strain and PEG concentration at least four replicates were performed. Inoculated tubes were incubated at 26 °C in an orbital shaker (200 rpm) for 48 h. For growth measurement, optical density (620 nm) was determined (Figueira et al., 2005). These results were used to calculate IC50 values, i.e. the concentration that inhibited growth by 50%.

2.6. Data analysis

Venn diagrams were built using the Venn tool from the Bioinformatics & Evolutionary Genomics, University of Ghent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Data from the production of siderophores, production of IAA, growth promotion of a non-legume through the emission of volatiles and

osmotolerance was submitted to univariate analysis, testing for differences displayed by bacteria isolated from the different plant species using Kruskal-Wallis One Way Analysis of Variance on Ranks and pairwise multiple comparison by Dunn's method (differences among plant species were considered significant when $p < 0.05$). The analysis was performed using SigmaPlot 11 (Systat Software).

Dendrograms from BOX-PCR fingerprints were built using GelCompar II (Applied Maths, Belgium) to calculate the Pearson correlation coefficient and analyze the clusters formed applying the unweighted pair group method with arithmetic mean (UPGMA).

Principal component ordination (PCO) was performed using Primer + Permanova (Primer-E, Plymouth). Data from the production of IAA, production of siderophores, VOCs influence in plant growth (*A. thaliana* leaf area and fresh weight), and bacteria osmotolerance was normalized and similarity matrix using Euclidean distance was calculated and used to perform PCO using the host plant species or the site as factors.

3. Results

3.1. Legumes diversity

The legume species present at each site differed. Of the 16 species harvested none was found in all four sites. Moreover, only one (*Ornithopus compressus* L.) was present at three sites. AT (1 species), VC (2 species) and MT (6 species) presented legumes that were exclusive to the site. On the other hand, legume species from AV were also present in AT (3), VC (2) and MT (1) (Fig. 2A).

3.2. Bacterial diversity

Molecular typing of bacterial isolates ($n = 180$) by BOX-PCR revealed 100 distinct profiles. One isolate representative of each profile was selected for 16S rRNA gene-based identification and further characterization. Most of the strains belonged to genera *Flavobacterium* (50%) or *Pseudomonas* (37%) and were isolated from different plant hosts at the four harvesting sites. The remaining strains (13%) belonged to different genera and were sporadic. From *Lysobacter*, *Variovorax*, *Achromobacter*, *Acinetobacter*, *Rhizobium*, *Stenotrophomonas* and *Paenibacillus* only one strain was obtained and from *Erwinia* and *Herbaspirillum*, two and four strains, respectively (Supplementary Table 1). From AT site, 34 strains were obtained, 11 from AV, 32 from MT and 23 from VC. The bacterial genera isolated from the root nodules of legumes at each site showed low variation (Fig. 2B), with two genera (*Flavobacterium* and *Pseudomonas*) being isolated from the four sites and accounting for 87% of the strains. However, some variation is observed among sites. MT is the most diverse site with 6 genera, AT and AV have 5 and VC 3 (see Supplementary Table 1 and Fig. 1B). Since the majority of strains belong to *Pseudomonas* or *Flavobacterium*, dendrograms were generated for these two genera to check if the site from which the bacteria were isolated influenced similarity (Supplementary Fig. 1A and B). However, these dendrograms show that there is no clear grouping of strains according to sites.

Comparing the two genera, most of *Flavobacterium* strains were isolated from *Vicia* (39%), *Medicago* (18%) and *Ornithopus* (16%) genera at the four sites. On the other hand, *Pseudomonas* strains were isolated from different plant genera at the four sites (Supplementary Table 1).

3.3. Bacteria plant growth promotion (PGP) abilities

The ability of strains to synthesize two important traits promoting plant growth was also analyzed in this study. Half of the strains (49.4%) synthesize IAA, although with distinct performances (0.02–18.6 μg/mL IAA/OD) (Supplementary Table 1). No statistical significance ($p > 0.05$) was found among the mean of IAA production of strains isolated from each legume species, albeit the host species from which bacteria were isolated influenced the ability of strains to produce IAA. In fact, the three strains isolated from *Trifolium repens* L. all synthesize IAA. The strains producing the higher concentrations of IAA (>14.5 μg/mL IAA/OD) were isolated from *Vicia* and *Medicago* genera mostly at MT (80%) (Fig. 3A). In contrast, strains from *Scorpiurus vermiculatus* L., *O. compressus* L., *Ornithopus sativus* Brot. and *Vicia benghalensis* L. did not synthesize IAA (Fig. 3A and B).

The ability to synthesize siderophores also varied among strains from different host species (Fig. 3C and D), despite the absence of statistically significant differences ($p > 0.05$). A high percentage (38–75%) of strains of *S. vermiculatus*, *Ornithopus pinnatus* (Mill.) Druce, *O. sativus* and *Vicia sativa* L. were able to synthesize siderophores (Fig. 3D). It was also in these host species that strains displayed the highest ability to produce siderophores and are all from VC site (Supplementary Table 1 and Fig. 3C).

The promotion of *A. thaliana* growth by the emission of bacterial volatiles was observed for many of the strains, with 47 of the strains yielding at least a 50% gain compared to control (no inoculation) when fresh weight is considered and 55 strains for total leaf area (Supplementary Table 1). Some of the strains lead to increments of up to 4-fold of fresh weight and up to 5-fold of total leaf area (Fig. 3E, G and I). Growth promotion through the emission of volatiles seems to be independent of

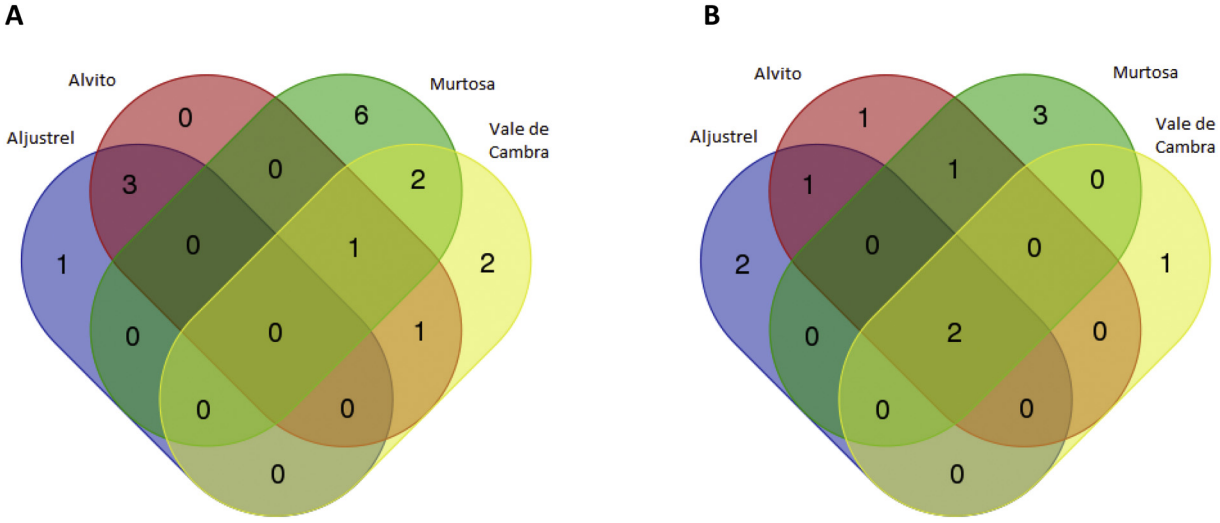

Fig. 2. Venn diagrams showing the legume species (A) and bacteria genera (B) distribution in the four harvesting sites: Aljustrel (AT); Alvito (AV); Murtosa (MT); and Vale de Cambra (VC).

the host legume species, since no statistically significant difference in *A. thaliana* leaf area and fresh weight was observed among strains isolated from different legume species ($p > 0.05$). However, the proportion of strains that promoted, inhibited or did not have an influence on *A. thaliana* varied among host species (Fig. 3F and H). For instance, considering leaf area (Fig. 3F), some host legumes such as *O. pinnatus* present strains with no effect, but also with the ability to promote or inhibit growth, while for the majority of strains isolated from a host species they either promoted growth or did not have an effect (Fig. 3F). Interestingly, when plant fresh weight is considered, only two host species present strains that inhibit *A. thaliana* growth (Fig. 3H), while strains from four host species inhibited leaf area (Fig. 3F).

3.4. Bacterial osmotolerance

Strain's osmotolerance was evaluated by growth in YEM supplemented with different PEG concentrations. Most strains (67%) presented IC_{50} between 8 and 15% PEG, and were considered moderately tolerant. Eighteen strains tolerated high PEG concentrations ($IC_{50} \geq 15\%$ PEG) and were considered tolerant, and 11 strains displayed IC_{50} lower than 8% PEG and were classified as sensitive to osmotic stress (Supplementary Table 1). The osmotolerance level of bacteria was not significantly different among genera ($p > 0.05$), with an IC_{50} between 11 and 14% PEG, except for *Herbaspirillum*. The average IC_{50} for this genus was 21.3% PEG and it included two of the most osmotolerant strains (IC_{50} of 25.8 and 26.4% PEG) (Supplementary Table 1).

The host species from which bacteria were isolated influenced their osmotolerance (Supplementary Table 1 and Fig. 4). Strains isolated from *V. benghalensis* (AT), *O. compressus* (AV, MT and VC), *Medicago lupulina* L. (MT), *Medicago littoralis* Rohde ex Loisel. (MT) and especially *Medicago polymorpha* L. (VC) presented high tolerance to osmotic stress, since 40 to 100% of the strains were classified as tolerant and the remaining moderately tolerant. Strains isolated from *Lotus corniculatus* L., *Vicia cordata* Hoppe. and *Vicia lutea* L. presented higher sensitivity, only including sensitive (9 to 33%) and moderately tolerant strains and all coming from MT and CV. Strains isolated from *Lathyrus clymenum* L. (AT and AV), *O. pinnatus* (MT), *Melilotus indicus* (L.) All. (MT), *T. repens* (MT), and *O. sativus* (MT and VC) are all moderately

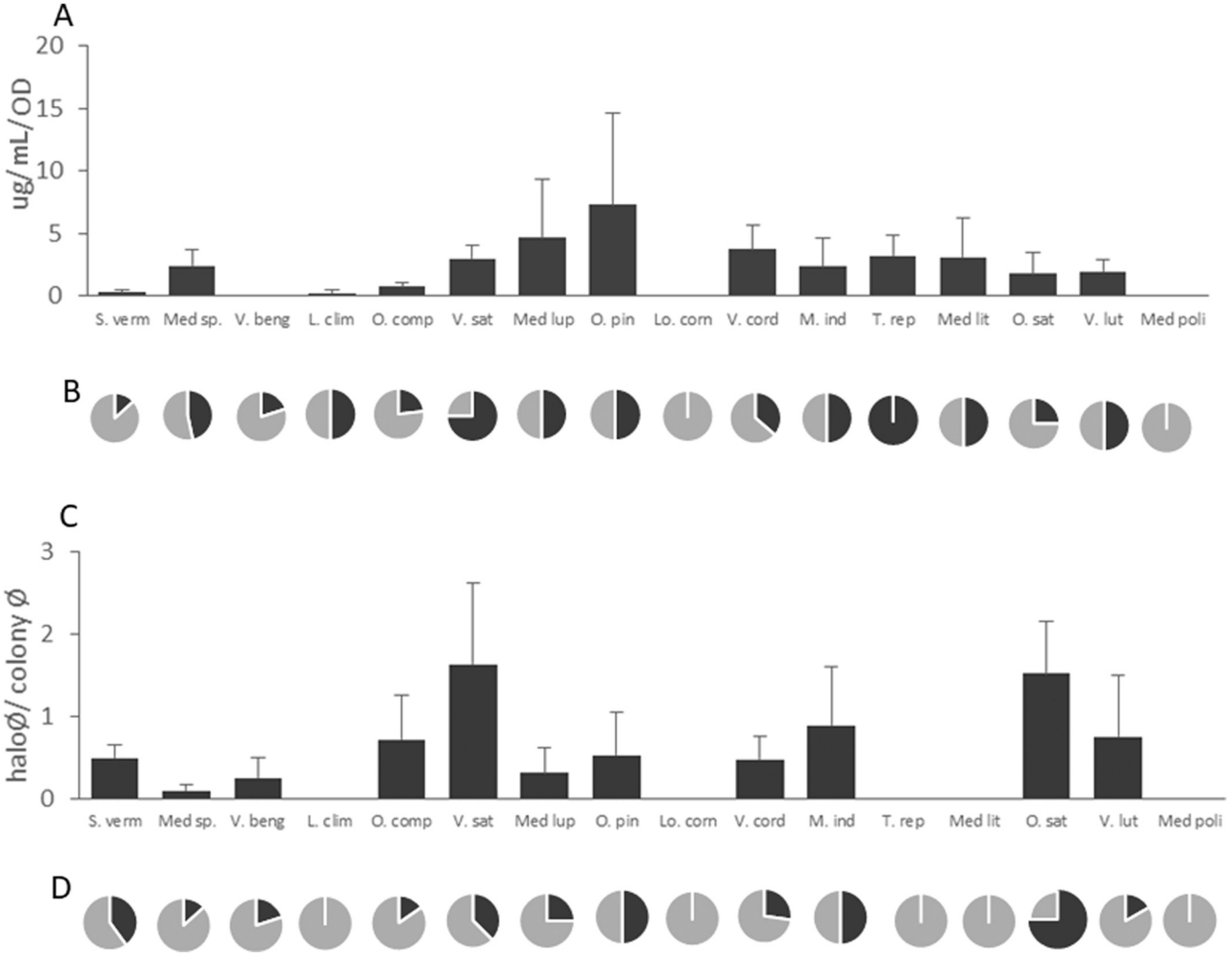

Fig. 3. Plant growth promotion traits of bacteria isolated from different host legume species: *Scorpiurus vermiculatus* (*S. verm*); *Medicago* sp. (*Med* sp.); *Vicia benghalensis* (*V. beng*); *Ornithopus compressus* (*O. comp*); *Vicia sativa* (*V. sat*); *Medicago lupulina* (*Med lup*); *Ornithopus pinnatus* (*O. pin*); *Lotus corniculatus* (*Lo. corn*); *Vicia cordata* (*V. cord*); *Melilotus indicus* (*M. ind*); *Trifolium repens* (*T. rep*); *Medicago litoralis* (*Med lit*); *Ornithopus sativus* (*O. sat*); *Vicia lutea* (*V. lut*); *Medicago polymorpha* (*Med poli*). (A) IAA produced by bacteria ((μg/mL)/optical density). (B) Proportion of strains able (dark grey) or unable (light grey) to synthesize IAA. (C) Siderophores (halo Ø/colony Ø). (D) Proportion of strains able (dark grey) or unable (light grey) to synthesize siderophores. (E) Leaf area of *Arabidopsis thaliana* exposed to bacterial strains, compared to the control. (F) Proportion of strains that promote (red), inhibit (orange) or do not influence (grey) *A. thaliana* leaf area. (G) Fresh weight of *A. thaliana* exposed to bacterial strains, compared to the control. (H) Proportion of strains that promote (red), inhibit (orange) or do not influence (grey) *A. thaliana* fresh weight. (I) Promotion of *A. thaliana* growth by bacterial volatile organic compounds. Example of a control plate (A) and inoculated plate (B).

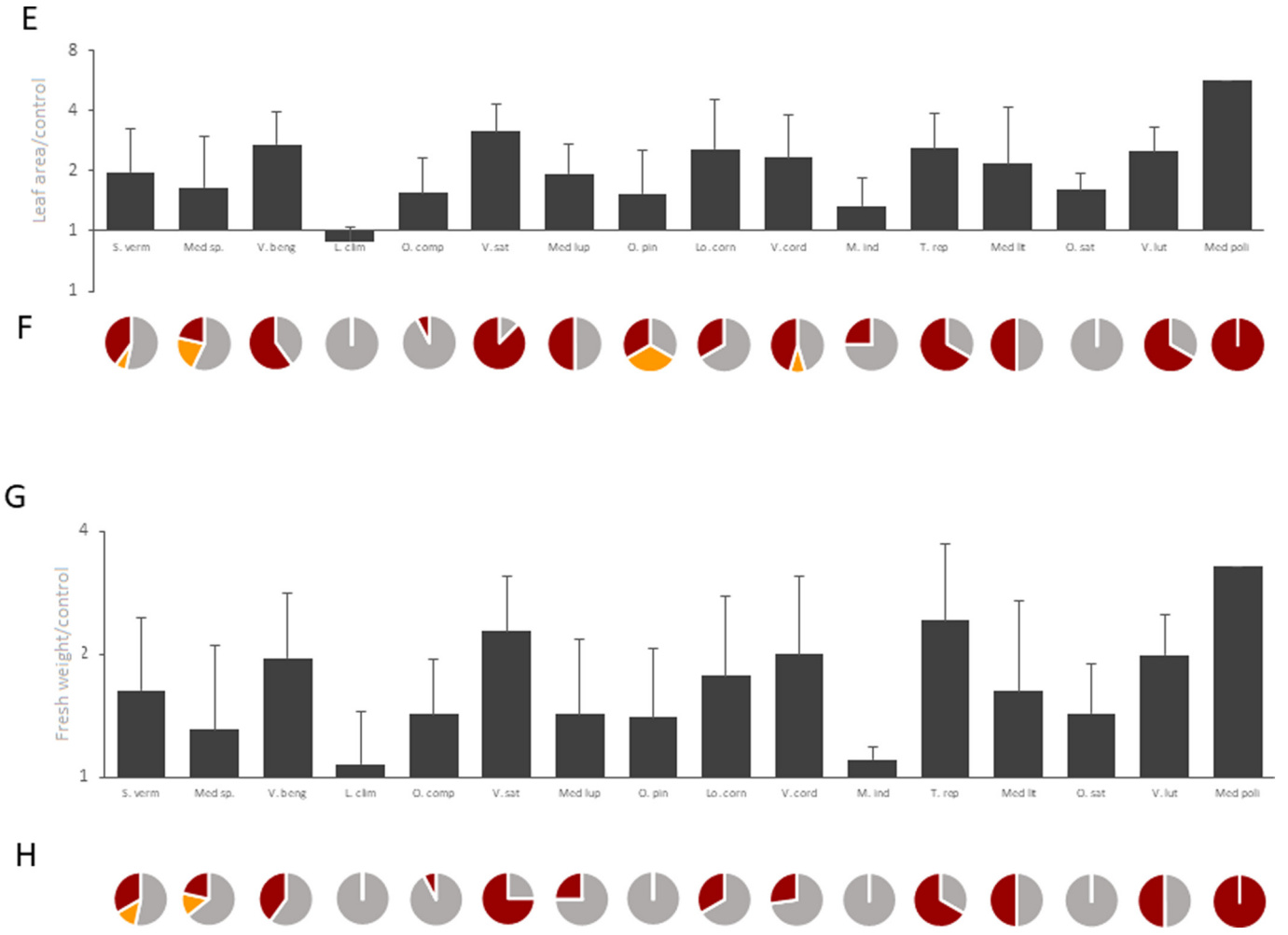

Fig. 3 (*continued*).

osmotolerant. Three plant species hosted strains with the three levels of tolerance and are from AT and AV (*S. vermiculatus* and *Medicago* sp.) or from AV and VC (*V. sativa*). The results evidence that strains osmotolerance is more associated with the host species than with location. In fact, the sites AT and AV had percentages of sensitive and tolerant strains similar to CV site. Most strains (88%) from MT are moderately tolerant and the number of sensitive strains was the lowest of the four sites. However, the strains displaying the highest osmotolerance (O3, S7 and P2) were isolated from the two southern sites (Supplementary Table 1).

3.5. Principal component ordination (PCO)

The PCOs of PGP abilities and osmotolerance using the host plant species as factor revealed that there was not an observable influence of host plant species in these bacterial properties (Supplementary Fig. 2). The influence of the site was also not observed (Supplementary Fig. 3).

4. Discussion

In the present study, we explored the diversity of bacteria harbored in the root nodules of legume species growing in wild areas of Portugal from distant geographical sites and distinct bioclimatic conditions. Since the different prevailing conditions between sites could have an impact on microbial diversity and performance, the isolation of bacteria from different sites and at the same time from different wild legume species was expected to yield different endosymbionts adapted to the conditions of the host when inside nodules and to the prevailing environmental conditions when in free living form. As expected, the different sites presented different legume species. However, a weak link of bacterial genera between the site of origin and the host species was observed. The genera isolated from the root nodules of the legumes collected in this study were all previously described as endophytic bacteria: *Flavobacterium* (Maheshwari, 2011), *Pseudomonas* (Maheshwari, 2011), *Erwinia* (Procópio et al., 2009), *Herbaspirillum* (Maheshwari, 2011), *Lysobacter* (Lòpez-Fernàndez et al., 2017), *Variovorax* (Maheshwari, 2011), *Achromobacter* (Dawwam et al., 2013), *Acinetobacter* (Maheshwari, 2011), *Agrobacterium* (Maheshwari, 2011), *Stenotrophomonas* (Maheshwari, 2011) and *Paenibacillus* (Costa et al., 2012). Several of these genera were already isolated from roots or other organs of legumes, including *Flavobacterium* (UmaMaheswari et al., 2013), *Pseudomonas* (UmaMaheswari et al., 2013) and *Paenibacillus* (Costa et al., 2012). Martínez-Hidalgo and Hirsch (2017) reported that among the most common non-nodulating endophytes found in legume nodules are *Azospirillum* (*Trifolium*, *Phaseolus*, *Vicia*, *Medicago*), *Variovorax* (*Crotalaria* and *Acacia*), *Pseudomonas* (*Vigna*) and *Paenibacillus* (*Medicago* and others). All these genera were also found in our study. Msaddak et al. (2017) found three genera in the root nodules of *Lupinus micranthus* Guss.: *Bradyrhizobium*, *Microvirga* and *Phyllobacterium*. Of the fifty isolates obtained by Msaddak et al. (2017), almost half belonged to *Microvirga* and *Phyllobacterium*. Both

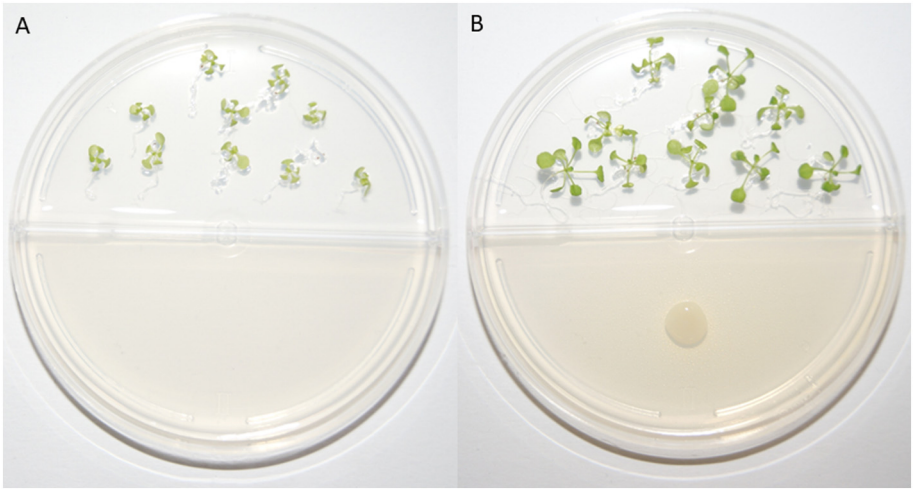

Fig. 3 (*continued*).

Microvirga and *Phyllobacterium* have been found in previous reports (Ardley et al., 2012; Flores-Felix et al., 2013). These reports, along with this study, evidence the diversity of bacteria that colonize legume nodules. Therefore, as more studies dealing with wild legumes species are published, the currently published known diversity of bacteria living inside legume nodules might increase. Bacteria that do not nodulate legumes can enter the nodule when rhizobia induce nodule formation (Ibáñez et al., 2009; Leite et al., 2017). Furthermore, a synergy between *Mesorhizobium* sp. and an endophytic *Pseudomonas chlororaphis* isolated from the root nodules of the legume *Sophora alopecuroides* L. was reported by Zhao et al. (2013). These authors described an increase in siderophore production, phosphate solubilization, organic acid production, IAA production and antifungal activity in vitro when the two different bacteria were co-inoculated (Zhao et al., 2013). Thus, the lack of knowledge about the role of non-nodulating bacteria, alone or in consortia, underestimates their potential benefits to the host plant.

The vast majority of the strains obtained in this study belong to the genera *Flavobacterium* and *Pseudomonas*. This result is in accordance with the fact that these genera are dominant in plant microbiomes (Piechulla et al., 2017). It is important to note, however, that many non-rhizobia bacteria apart from *Pseudomonas*, *Flavobacterium* and the remaining identified in this work have been reported in legume nodules and consequently it is unlikely that these genera dominate legume nodules in general. Examples include *Micromonospora* (Trujillo et al., 2010), *Azospirillum* (Cassán and Diaz-Zorita, 2016) and *Bacillus* (Zakhia et al., 2006). Regarding *Micromonospora*, the absence of these strains in our work could be due to the fact that it is a slow grower (3 weeks of incubation at 28 °C) (Trujillo et al., 2010). Nonetheless, in our work *Flavobacterium* and *Pseudomonas* were found independently of the host legume species. It has been accepted for long that there is a molecular specificity between the two partners of the symbiotic relationship, that is the bacteria and the legume species (Somasegaran and Hoben, 1994), yet this knowledge is mainly based on the symbiosis between rhizobia and crop legumes. Unlike crop species, wild legumes seem to be more promiscuous in the interaction with rhizobia (Mutch and Young, 2004). Based on our results, this promiscuity seems to extend to non-rhizobial bacteria. A recent study by Hartman et al. (2017) reported abundant *Pseudomonas* isolates in the *Trifolium* root microbiome.

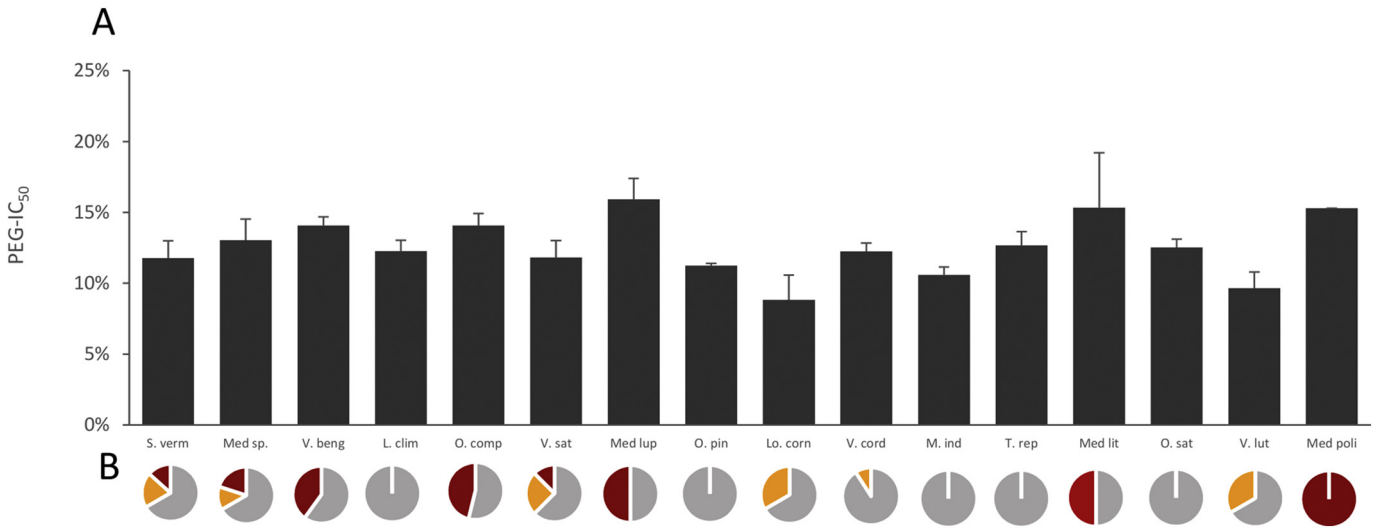

Fig. 4. Osmotolerance of bacteria isolated from different host legume species: *Scorpiurus vermiculatus* (*S. verm*); *Medicago* sp. (*Med* sp.); *Vicia benghalensis* (*V. beng*); *Ornithopus compressus* (*O. comp*); *Vicia sativa* (*V. sat*); *Medicago lupulina* (*Med lup*); *Ornithopus pinnatus* (*O. pin*); *Lotus corniculatus* (*Lo. corn*); *Vicia cordata* (*V. cord*); *Melilotus indicus* (*M. ind*); *Trifolium repens* (*T. rep*); *Medicago litoralis* (*Med lit*); *Ornithopus sativus* (*O. sat*); *Vicia lutea* (*V. lut*); *Medicago polymorpha* (*Med poli*). (A) Concentration of PEG (%) inducing 50% growth inhibition (IC50). (B) Proportion of strains with different osmotolerance levels: Sensitive (IC50 < 8% PEG)-yellow; moderately tolerant (8% ≤ IC50 < 15% PEG)-grey; tolerant (IC50 ≥ 15% PEG)-red. Values are means of 3 replicates ± standard deviation.

In the same study, *Flavobacterium*, *Stenotrophomonas* and *Paenibacillus* were also observed (Hartman et al., 2017). *Flavobacterium*, *Pseudomonas* and *Paenibacillus* were the dominant fraction of non-rhizobia isolates found inside root nodules of native legumes in a study in Flanders (Meyer, 2011). Both *Flavobacterium* and *Pseudomonas* have been reported as capable of inhibiting plant pathogens (Vijayabharathi et al., 2016). Some *Pseudomonas* strains are plant growth promoting rhizobacteria used as inoculants to promote plant growth and alleviate stress in plants (Ahemad and Kibret, 2014). *Pseudomonas* were also isolated from the rhizosphere of legumes, such as chickpea (*Cicer arietinum* L.) and green gram (*Vigna radiata* (L.) Wilczek) (Sindhu and Dadarwal, 2001). Ibáñez et al. (2009) reported that *Pseudomonas* entered the nodules of peanut (*Arachis hypogaea* L.) in an opportunistic manner. Nonetheless, these authors found that these *Pseudomonas* strains improved plant yield. Moreover, some *Pseudomonas* were described as diazotrophic (Santi et al., 2013). Regarding *Flavobacterium*, negative effects on the growth of *Trifolium pratense* L. were reported, which could be counteracted when other bacteria were co-inoculated (Hartman et al., 2017). However, in our study some *Flavobacterium* strains displayed high ability to produce IAA and to promote *A. thaliana* growth through the emission of VOCs. Soltani et al. (2010) and Tsavkelova et al. (2005) also reported *Flavobacterium* as a IAA producer and a bacteria genus promoting plant growth (Ahemad and Kibret, 2014).

The fact that in this study non-rhizobial bacteria were found in root nodules of different wild legume species collected from four distant geographical sites, suggests that nodule colonization by non-rhizobial bacteria is not an anecdotal phenomenon but rather a common occurrence, at least in legumes that are growing in land not used for agricultural purposes. It could be that in agricultural soils the isolation of these non-rhizobial bacteria is less frequent, due to the presence of different species usually cultivated in different conditions (fertilized and irrigated soils). The nutritional conditions provided to plants and from the plants to the microsymbionts can also differ. In agricultural areas, due to the growth of crop legumes in monocultures, large increases in rhizobial populations with higher affinity to the host occur (Hirsch, 1996; Kucey and Hynes, 1989). In wild areas, a high diversity of both legume and non-legume plants co-habit, and these non-rhizobial bacteria can play important roles as endophytic bacteria, providing benefits to plants, which can be crucial in the predominant conditions of these sites, where there is no input of fertilizers, irrigation and pesticides as in agricultural fields, and thus plants are more vulnerable to nutrient deficiencies, water scarcity and infection.

Approximately half of the strains obtained in this study are IAA producers, and therefore have the potential to alleviate legumes suffering from drought-induced stress (Hussain et al., 2014). This can be put at risk if the bacteria cannot survive in an environment subjected to drought. Most of the strains in this work are moderately tolerant to PEG, although strains range from sensitive to tolerant. This information is important when selecting PGPR to be used as inoculants in areas frequently subjected to drought. Although inside the nodule some level of protection might be offered by the plant, the bacteria must be able to survive in the soil prior to infection.

The low specificity between the non-rhizobial endophytic bacteria and the host legume species observed in our study is an important feature both from an agronomical and an ecological point of view. If the strains selected as inoculants for legumes do not present a narrow specificity between the bacteria and the legume crop species, and instead bacteria are able to colonize a broad range of hosts, than strains with interesting PGP capabilities can effectively be used to enhance growth of different legume and non-legume species. In fact, the PGP abilities which were evaluated in this study (siderophores, IAA and volatiles capable of promoting the growth of *A. thaliana*) show that these bacteria can provide benefits to plant productivity which are not restricted to the host from which they were isolated. This becomes especially apparent with the airborne growth promotion of a non-legume, *A. thaliana*, by these strains (isolated from legumes). Moreover, bacterial VOCs that are able to promote plant growth might be beneficial in the field not only for their host, but also to surrounding plants. However, this might also present the disadvantage of promoting the growth of weeds. Bacteria from the genera *Bacillus*, *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Arthrobacter* and *Chromobacterium* have been reported previously as emitting VOCs with effects on *A. thaliana*, *Medicago sativa* L., *Nicotiana tabacum* L., *Triticum aestivum* L. or *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Bailly and Weisskopf, 2012). Apart from effects observed from strains of the already reported bacterial genera (e.g. *Pseudomonas* and *Stenotrophomonas*), in this study *A. thaliana* growth promotion by several strains of *Flavobacterium*, *Herbaspirillum*, *Variovorax*, *Achromobacter*, *Erwinia*, *Acinetobacter*, *Agrobacterium*/*Rhizobium*, and *Paenibacillus* was shown (see Supplementary Table 1), thus extending the known range of bacterial genera capable of promoting plant growth through the emission of VOCs. Moreover, based on our results, these benefits are not restricted to a geographical area and its prevailing conditions, neither to a bacterial genus nor host legume species. This approach reduces the effort for the selection of effective strains which instead of being specific for a reduced set of plant species, can have a more broad use. From an ecological point of view, this issue is also important since legume species colonizing the same site can all benefit from the advantages provided by the most effective strains present in each site, be it growth improvement, resistance to infection or drought tolerance.

5. Concluding remarks

Our study showed that other bacteria besides rhizobia can be widely found in wild legumes nodules, particularly *Flavobacterium* and *Pseudomonas*, and might influence the growth of legumes and therefore should be studied so that they can be used in concert to improve legume and non-legumes productivity (Zahran, 2001). This becomes specially important as new mechanisms of PGP are found, such as the emission of bacterial volatiles that can have multiple benefits for plants (Piechulla et al., 2017), and which can strengthen the biotechnological potential of PGP bacteria to be used in the improvement of legume productivity while keeping environmental sustainability in mind.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.399.

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Conflict of interest

The authors declare no conflict of interest.

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